Studies concerning Chemistry and Immunological Properties of Pneumococcus

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STUDIES ON ANTIGENIC DISSOCIATION:

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Investigations on the immunological relationships of the carbohydrate and protein of Pneumococcus clearly indicate that the bacterial cell functions as a complex antigen, the components of which are subject to dissociation. Moreover, the nature of the immune response of animals experimentally infected or artificially immunized with Pneumococcus is conditioned by principles which govern the rate and extent of antigenic dissociation under given conditions. Cell disintegration, whether brought about by physical (freezing and thawing), by chemical (bile and alkalis), or by enzyme action (autolysis), is always accompanied by partial or complete loss of the capacity of the dissolved material to stimulate type-specific antibodies. Although in cell solutions the specific polysaccharide (haptene) exists free and unaltered in specific reactivity, in this form it is completely devoid of the power to provoke antibody production. Since in the natural state in which it is present in the intact cell this same carbohydrate substance is not only highly specific but extraordinarily efficient as antigen, and since it loses its antigenic property to a greater or less extent whenever cell dissolution occurs, the conclusion seems inevitable, that cell disintegration is accompanied by antigenic dissociation, in which process the soluble, specific substance is split off from some other constituent with which it forms the dominant type-specific antigen of the cell as a whole. It likewise follows that morphological integrity is essential to the fullest expression of the antigenic value of the cell. Although by reason of antigenic dissociation solutions of pneumococci may fail completely to elicit the type-specific antibodies, such solutions always stimulate the formation of antibodies which are qualitatively different in

that they react only with the protein of the cell without regard to type-specificity; that is, they are species but not type-specific. An immune serum containing only the anti-protein immune bodies precipitates solutions of the isolated protein regardless of its type derivation and agglutinates all degraded "R" cells which by cultural or chemical means have been stripped of the type-specific substance. These same protein antisera, however, are not reactive with encapsulated pneumococci or with pure solutions of specific bacterial polysaccharides. Furthermore, such sera afford no protection to mice against infection with virulent organisms of the fixed types. In other words, pneumococcus protein antibodies are unrelated to the type-specific mechanism of the cell and pertain only to the broader protein-antiprotein reactions of the species. These facts admit of at least two interpretations.

From observations which have been repeatedly confirmed experimentally, it is evident that both the carbohydrate and protein of pneumococcus share in the immunological mechanism of the cell, either as two distinct and independent antigenic systems, or as a single complex in which carbohydrate and protein together form one antigenic unit. If these two constituents, both integral parts of the cell as a whole, be considered as forming two separate and unrelated antigens, then that system comprising the carbohydrate fraction may be referred to as the ectoplasmic, capsular or type antigen, and the other, involving the protein of the cell body, may be spoken of as the endoplasmic, somatic or species antigen. Since the specific polysaccharide (haptene) is by itself non-antigenic, it follows that in the system of which this material forms a part the carbohydrate

does not exist as such but in combination with some other substance with which it forms an antigenic complex each component of which is separately inert. That is, once this union has been disrupted the antigenicity of the whole is lost and only the specific antibody binding-property of the dissociated haptene remains. This type-specific substance of the cell has now been chemically identified as a polysaccharide and can be recognized in the system after dissociation by its specific reactivity with the type antibodies induced by the intact cell in which the two components still remain undissociated and hence antigenic. The nature and properties of the factor in this primary system which enters into combination with and confers antigenicity upon the specific polysaccharide are unknown. According to the conception of "double antigen", the second and equally independent antigenic system of the cell would consist solely of the bacterial protein. This substance, constituting the larger part of the cell body, functions antigenically precisely as do the isolated nucleoprotein and the decapsulated "R" cells in stimulating only the anti-protein antibodies which characterize the species.

A simpler and perhaps more likely interpretation of the dual antigenic function of the intact cell is the concept that the bacterial polysaccharide and protein exist not as individual members of separate systems, but as a single carbohydrate-protein complex, the specific antigenicity of which is determined by the presence of the type-specific polysaccharide. In other words, the bacterial cell as an antigenic unit consists of a combination of nucleoprotein and carbohydrate in which the latter determines the type-specificity of the whole. This compound antigen is dissociable and the ease with which the linkage

between the two cellular constituents is disrupted varies with each of the fixed types. When dissociation occurs, as it does to a greater or less extent whenever dissolution of the cell takes place, the specific (polysaccharide) haptene is split off leaving only the protein fraction which then functions as a secondary antigen. This conception is supported by experimental evidence; although solutions of bacterial cells, in which complete dissociation has occurred, contain the total content of body substance present in the formed elements, these solutions are devoid of the property of stimulating the type-specific antibodies which characterize the immune response to the intact organisms. Immunization with these solutions results only in formation of the secondary antiprotein antibodies.

The evidence presented thus far concerns antigenic dissociation in vitro, as revealed by differences in the antibody response to intact and dissolved pneumococci. There is also considerable evidence that similar dissociation goes on in the animal body after the introduction of the whole cell. The most striking example of the occurrence of this phenomenon in the animal body is the character of the immune response in rabbits to immunization with Pneumococcus Type III. Repeated injection of encapsulated Type III pneumococci fail in the great majority of instances to elicit any type-specific antibodies; the serum of immunized rabbits neither agglutinates the encapsulated cells, nor precipitates the homologous specific substance, nor affords protection to mice against infection with the virulent organisms of Type III. Indeed, such sera, if tested solely for the presence of antibodies of the type-specific variety, would be considered devoid of all immune bodies. However, these sera are rich in antiprotein antibodies; they

not only precipitate protein-containing solutions of pneumococci, but agglutinate the decapsulated bacteria ("R" forms). This result can only be intrepreted as evidence that in the animal body the same or a similar process of dissociation goes on as that which occurs with disruption of the cell in vitro. These observations, now supported by experimental evidence, furnish a basis for understanding the difficulties encountered in attempts to produce an efficient antiserum for Type III pneumococcus. Indeed, the principle underlying this phenomenon is perhaps applicable to the other types as well. It seems not unlikely that the relative differences in the potency of immune sera, the effectiveness of which is greatest in Type I, less in Type II, and least in Type III, may be referable to differences in the ease of dissociation of the specific antigen complex in each type. It is interesting in this connection to recall the fact that, although there is a progressive increase in the amounts of soluble specific substance elaborated by Types I, II, III in the order named, there is a corresponding decrease in the antigenic efficiency of the three types. The specific polysaccharides in each instance are known to be chemically distinct substances and these differences in chemical constitution may account not only for the specificity of each, but may also determine the rate and completeness with which the antigenic complex of each undergoes dissociation. According to this view, the most efficient antigen is the one least easily dissociable; that is, the antigenic potency of a given type is inversely proportional to the rate and extent of dissociation.

Like all antibacterial processes in the body, the phenomenon of antigenic dissociation is determinedby two independent but interrelated groups of factors; those pertaining to the animal body and those

relating to the micro-organisms.

Factors relating to the animal: These comprise the antibacterial properties which are possessed naturally by the animal organism and which together constitute the vaguely defined quality of bodily resistance. Since in the case of the Type III pneumococcus the rabbit can tolerate with impunity ten million times the dose fatal to a mouse, it is apparent that this result is determined by differences in the normal resistance of the two animals to the same organism. However, to say that the rabbit possesses natural resistance to infection with Pneumococcus mucosus, and that the mouse does not possess this property, merely restates the problem and fails to explain the facts. If, on the other hand, the evidence in favor of the dissociation hypothesis be accepted, and this newer concept of the bacterial cell be applied to processes occurring within the body, then differences in animal resistance may be interpreted in terms of this dissociation process. There is immunological evidence that dissociation involves an injury to the capsular mechanism of the cell, whereby the potentially virulent organism is reduced to a form resembling in antigenic and infective properties those cells which have been degraded "R" forms by growth in vitro. Just as degradation of the encapsulated organism by cultural methods is accompanied by loss of virulence and type-specificity, so the injury inflicted on the bacteria by the tissues of the resistant animal results in a loss of invasiveness with the liberation of the common protein antigen. Both results are the expression of the same phenomenon; injury to the capsular mechanism not only exposes the denuded cell to phagocytosis, thus accounting for the lack of infection, but this same injury, by dissociating the "S" substance, discloses the undifferentiated protein of

the cell, thus accounting antigenically for the presence of only anti-protein antibodies in the blood serum of the resistant animals. Although resulting from the same process, the survival of the animal is not necessarily dependent upon the concomitant development of protein immunity. The formation of the antiprotein immune bodies is in all likelihood merely a secondary reaction dependent upon the presence of the dissociated protein. This view seems the more likely since purely antiprotein sera do not confer passive protection on animals susceptible to infection.

Although the nature of the bacterial injury which affects the invasive and antigenic function of the cell is, as yet, unknown, the result is two-fold; first, the survival of the animal in the presence of a potentially virulent organism, and second, the development of a purely antiprotein immunity by a cell potentially capable of stimulating the type-specific, anti-carbohydrate antibodies. The loss of virulence and the depreciation of the antigenic value of pneumococci within the body of the resistant animal are, in outcome at least, analogous to the degradation of these same two functions which is induced by growth in an unfavorable environment.

Animals of different species and individual members of the same species vary in the ability to inflict the cell injury which leads to antigenic dissociation. The presence of this property in the tissues determines the animals' resistance to a particular micro-organism, as in the case of the rabbit to Type III Pneumococcus, and the absence of this property determines the animals' susceptibility, as in the case of the mouse to the same strain. Quantitative differences would account for the variations observed in the susceptibility of individual animals. Natural resistance, whereby an animal disarms the invading pneumococci

by stripping the capsule, thus robbing the cell of infectivity and type-antigenicity, is an innate quality which differs from acquired immunity in that the serum of the resistant animal is devoid of type-specific antibodies and fails to confer passive protection against infection in susceptible animals.

Reference has already been made to the relative differences in the potency of immune sera of Types I, II, and III. The reason for this progressive decrease in potency of the respective antisera was ascribed to differences in the ease of dissociation of the homologous antigen. According to this view, then, the amount of antigenic dissociation occurring both before and after the injection of the immunizing material into the animal body determines the proportional content of the type-specific and the antiprotein antibodies in the serum. Conversely, the relative titre of these two qualitatively different antibodies serves as an index of the comparative ease with which the specific antigenic complex of a given type undergoes dissociation. In animals of the same species the factors of natural resistance which result inantigenic dissociation are not equally effective against all types of pneumococci; that is, what has been described as the reaction of the normal rabbit to Type III does not obtain in the case of pneumococcus Type I.

Factors Relating to the Micro-organism: Differences in the immune response of the same animal to pneumococci of different types suggests the operation of factors peculiar to the micro-organisms themselves in addition to those previously described as relating to the animal body. In accordance with the dissociation theory, these bacterial properties are referable to differences in the ease with

which the antigenic complex of the specific types undergoes dissociation in the presence of the resistance factors of the host. That is, dissociation, like specificity itself, is dependent upon the particular chemical structure of the complex antigen of each type. Since the bacterial polysaccharides, which define specificity, are separate entities as distinctive in their chemical properties as they are specific in their serological reactions, the factors determining antigenic stability of any given type of pneumococcus are inherent in the chemistry of the cell itself. For instance, in the case of Type III pneumococcus, the least stable of all type antigens, the linkage of the carbohydrate complex is more easily disrupted and complete dissociation of the type-specific polysaccharide leaves only the protein to function as antigen. This intrepretation explains the hitherto inexplicable fact that immunization of rabbits with Pneumococcus Type III results only in the formation of antiprotein antibodies. In the case of pneumococcus Type I, on the other hand, the more stable union of the antigenic complex resists cleavage and the intact antigen functions in the stimulation of type-specific antibodies. The potency of antipneumococcus serum as measured by the content of type-specific antibodies is conditioned, therefore, by a balance between the factors of the animal body which bring about antigenic cleavage and those properties of the bacterial cell which determine the stability of the specific antigenic complex of each type.

It is interesting to apply the same concept to the problem of bacterial virulence. When an animal does not possess in adequate amount the factors of natural resistance or when even in their presence the bacterial cell is so constituted as to make difficult or impossible

the dissociation of the cellular components so that the Pneumococcus retains its capsular mechanism unimpaired, is it not possible that this natural or acquired resistance on the part of the micro-organism is the dominant factor in the phenomenon of bacterial virulence? And, when the virulence of an organism is enhanced by rapid and repeated passage through animals for which it originally possessed little virulence, is it not possible that this property consists in the acquisition of an increased resistance on the part of the cell to the dissociation factors of the animal;- an adaptation or form of "tissue fastness" acquired by the micro-organism?

According to this view, the lack of virulence of Type III Pneumococcus for normal Rabbits, and the absence of the type-specific antibodies in the serum of resistant animals would each be referable to the same cell injury, that is, to the process of cell dissociation. If this interpretation is correct, then by rabbit passage the pneumococcus mucosus should acquire increased resistance to dissociation and the acquisition of this "tissue fastness" should be accompanied by increased virulence and the capacity to stimulate the type-specific (anti-S) antibodies which is a function of the undissociated antigen of the intact cell. In this hypothesis perhaps lies the explanation of the fact empirically discovered, that the more virulent the organism used for immunization the more potent and specific is the antiserum; a result dependent not merely upon the fact that the virulent cell elaborates more of the type-specific substance, but that with the accession of virulence, the cell becomes increasingly more resistant to cleavage, thus conserving in its effective state the dominant type-specific antigen.

Application of the Principles of Antigenic Dissociation to Production of Antipneumococcus Serum: (Drs. Avery and Julianelle).

In the preceding discussion of the theory of antigenic dissociation attention has been drawn to the significance of this phenomenon in the production of potent antipneumococcus serum. Since the evidence thus far available clearly indicates that the most efficient antigen is the one least easily dissociable and that the antigenic potency of any given type of pneumococcus is inversely proportional to the rate and degree of dissociation, attempts have been made to increase the type-specific antibodies in immune sera by special methods designed to prevent dissociation of the type-specific antigen.

Intact, encapsulated pneumococci, bearing their full complement of type-specific carbohydrate, have been treated with various chemical reagents with the hope of "fixing" the cell so as to conserve intact the effective antigenic complex by rendering it less susceptible to those factors which bring about dissociation. By reason of the chemical nature of the type-specific carbohydrate of Type I, which contains an amino sugar group, formol was chosen as "fixative". The method employed and the results obtained are as follows:- Method: Young cultures of an "animalized" strain of Type I pneumococcus were grown in plain broth for 8-10 hours. The culture was divided into two equal parts. One portion was heated at 56° C. for one hour and the other half was killed by the addition to the culture fluid of formaldehyde in final concentration of 0.2 per cent.

Two series of rabbits were immunized according to the method of Cole and Moore, that is, 1 cc. of bacterial suspension was given intravenously every day for one week followed by a rest period of one

week, until the animals had received three courses of injections. One group of rabbits was given injections of heat killed culture, while a second group was given the nonheated, formalinized culture. Before each course of injections blood was drawn from the ear and the serum tested for the presence of type-specific (anti-"S") and species specific (anti-"P") agglutinins.

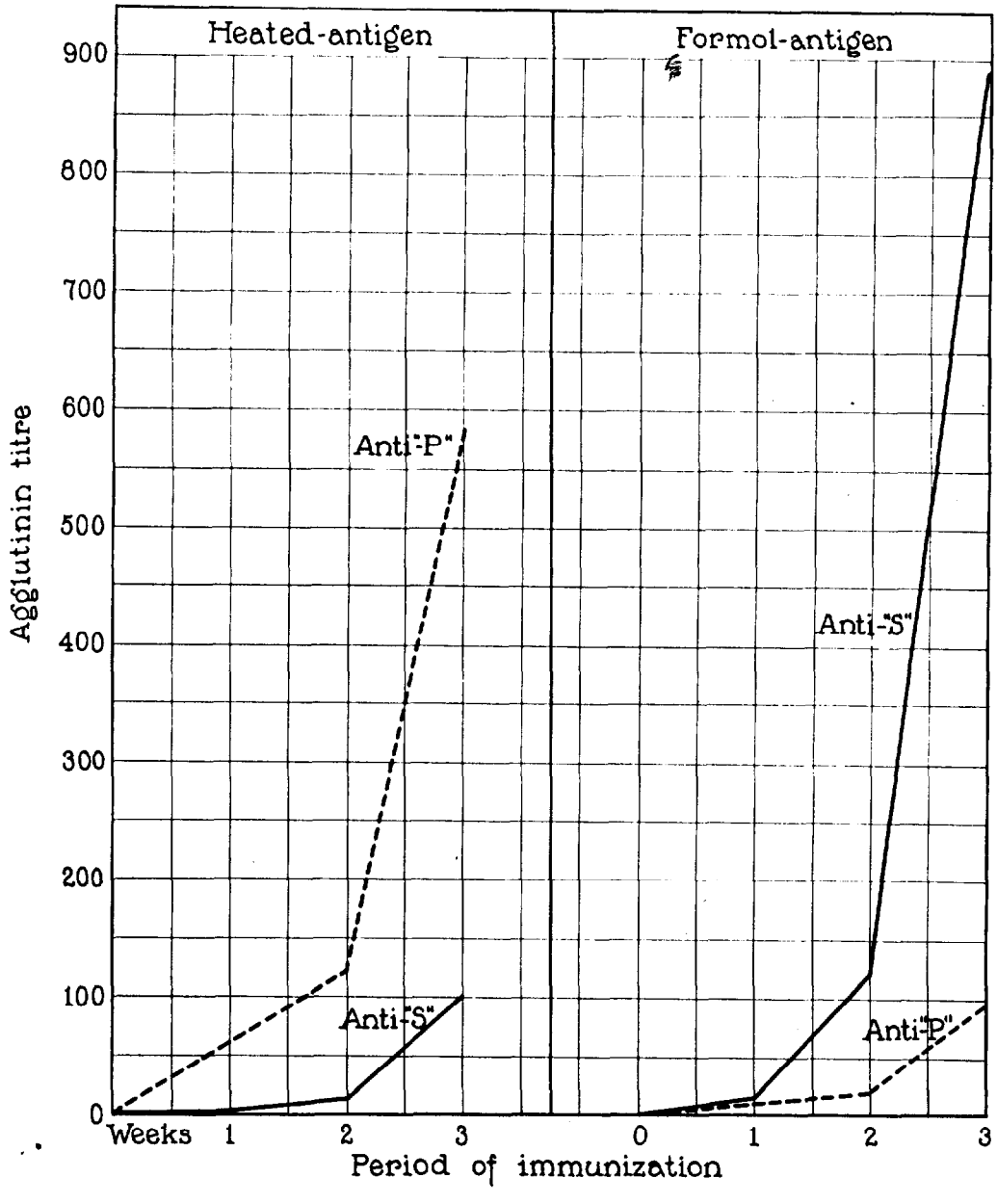
The accompanying protocol shows the results of the immunization.

T A B L E I

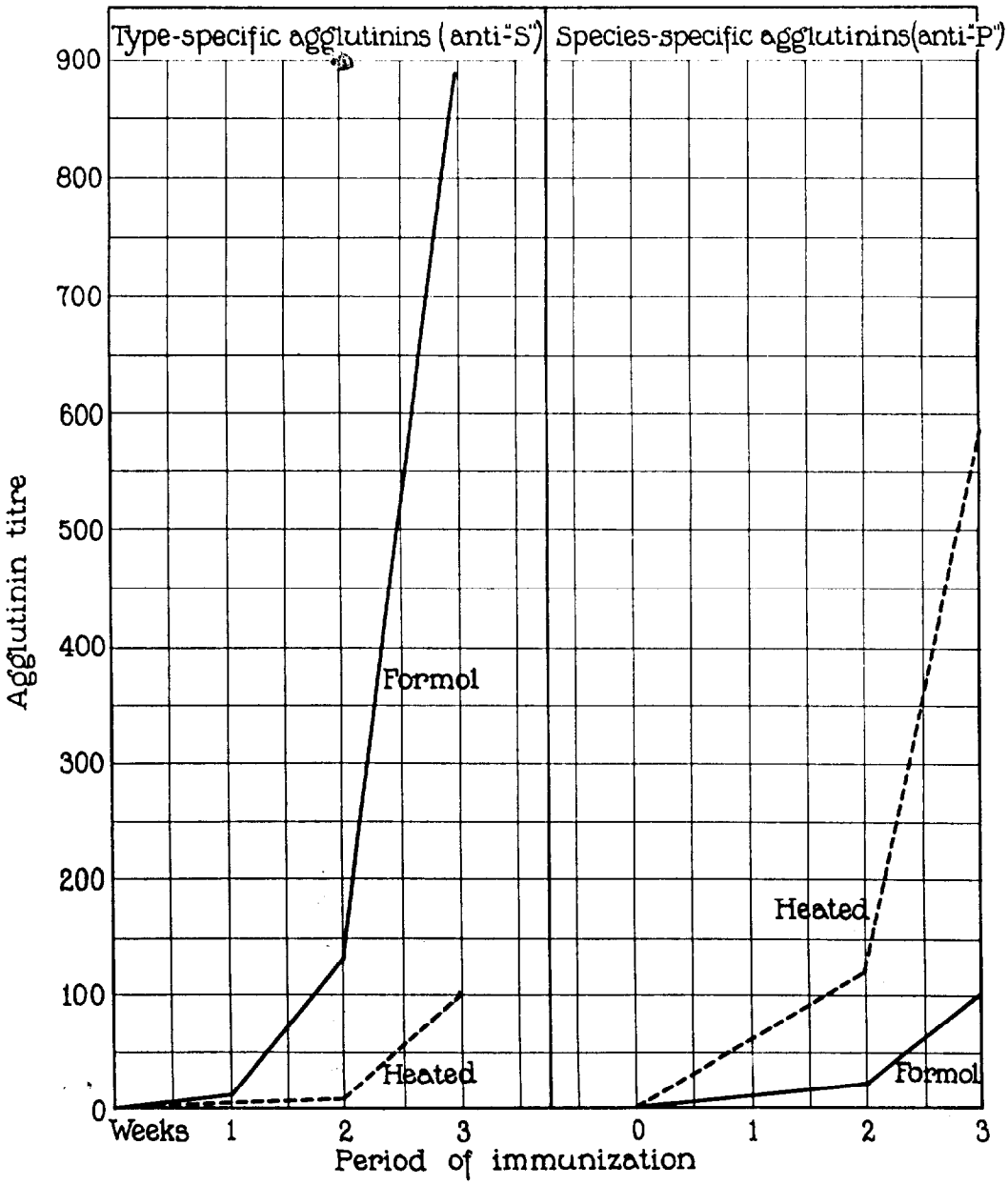
	Type Specific Antibodies.			Species Specific Antibodies		
	Rabbit No.	Heat killed antigen	Formalinized antigen	Rabbit No.	Heat killed antigen	Formalinized antigen
After 1 course of injections	1	Negativ	1:8	7	-	-
	2	"	1:8	8	-	-
	3	"	1:4	9	-	-
	4	"	1:16	10	-	-
	5	"	1:16	11	-	-
	6	"	1:16	12	-	-
After 2 courses of injections	1	1:5	1:160	7	1:80	Negative
	2	1:10	1:160	8	1:160	1:40
	3	1:10	1:180	9	1:80	Negative
	4	1:10	1:180	10	1:80	1:40
	5	1:20	1:160	11	1:160	1:40
	6	1:10	1:160	12	1:160	1:20
After 3 courses of injections	*1			*7		
	2	1:80	1:640	8	1:640	1:160
	3	1:160	1:1280	9	1:640	1:160
	4	1:40	1:640	10	1:640	1:80
	5	1:80	1:640	11	1:640	1:40
	6	1:160	1:1280	12	1:320	1:40

* Rabbits #1 and #7 were found dead

Ratio of anti-"S" to anti-"P" antibodies in sera of rabbits immunized with heat-killed and formol-treated pneumococci (type I)



Comparison of the immune response in rabbits
to heat-killed and formol-treated pneumococci (type I)



The results are summarized in Table I and graphically presented in Figures 1 and 2. Formalized cells give rise to type-specific antibodies more promptly and the titre at the end of the period of immunization averages over eight times the titre induced by the heat-killed vaccines. Moreover, the striking difference in the proportional content of the type-specific and antiprotein antibodies is evident in the inverse ratio of these two qualitatively different antibodies. (See Figures 1 and 2). If these differences are interpreted in terms of antigenic dissociation it seems not unlikely that methods, based on the chemical requirements of each specific type, may be found which will serve to inhibit the rate and degree of dissociation and thus tend to "stabilize" these complex antigens. The increased potency of the formalized antigen of Type I pneumococcus, a method not applicable to the organisms of Type II and III, is at least suggestive that with appropriate reagents similar results may eventually be obtained with the other fixed types.

Observations on the occurrence of the Specific Types of Friedlander's Bacillus in Disease. (Dr. Julianelle). In the preceding report three specific types and a heterogeneous group were described among the Friedlander bacilli. The encapsulated forms were type specific, but by artificial methods they were rendered capsule-free and were then found to be only species specific. Recently, nine strains of the organism have been referred to us for typing. Six of these strains were found to fall in Type A, one in Type B, and two in Type C. Three of these cultures were associated with chronic human infections and all were found to be of a composite nature - that is, they included both "S" encapsulated and "R" capsule-free, forms.

In four instances, the organisms were isolated from in-

dividuals suffering from lobar pneumonia due to Friedlander's bacillus. In a fatal case of Friedlander pneumonia in this hospital the opportunity was offered for studying the type specific precipitin reaction in the urine. The reaction to type was prompt and occurred in a dilution of urine of 1:16 on the second day, and 1:64 on the third day of the disease when the patient died. Thus confirmation was gained of Blake's observations of a specific precipitin reaction in one case of pneumonia due to Friedlander's bacillus and of our own observations of a similar reaction in experimentally infected rabbits.

The following table shows the distribution of the specific types of Friedlander bacilli summarized to date.

The occurrence of Specific Types of Friedlander's Bacillus.

Total number of Strains studied	Type A	Type B	Type C	Group X
39	21	7	5	6
Type A - 21 strains:	19 from lobar Pneumonia, 1 from extirpated adenoids, 1 from cystitis.			
Type B - 7 strains:	3 from guinea pigs (pneumonia), 2 from lobar pneumonia (human), 2 from horses (genito-urinary infection)			
Type C - 5 strains:	2 lobar Pneumonia, 1 from antrum infection, 2 source unknown.			
Group X - 6 strains:	5 lobar Pneumonia, 1 from faeces (Pellagra)			

Although the total number of strains of Friedlander bacilli studied so far is too small to furnish conclusive data on the distribution and relative frequency of occurrence of the specific types in infections, it is, nevertheless, interesting to observe that of 28 isolated from lobar pneumonia in man 23 or 82% belonged to one or other of the three fixed

types. The frequency of Type A infection in Friedlander pneumonia in human beings is shown by the fact that this type was isolated from 19 (68%) of 28 cases studied. From three different epidemics of Friedlander pneumonia in guinea pigs, three strains were isolated all of which belonged to Type B. Moreover, 5 out of the 7 strains belonging to this type were from animal sources, the remaining two being associated with pneumonia in man.

A Study of Complex Antigens. (Dr. Julianelle). Previous studies from this laboratory have revealed the interesting fact that organisms of widely different species may exhibit striking reciprocal immunological reactions. This was found to occur in the case of Friedlander's bacillus (Type B) and Pneumococcus (Type II). Chemical studies had shown previously that the soluble specific substances of the two organisms possess certain chemical properties in common, and it was thought at the time that the mutual immunological behavior of the two strains was referable to the chemical similarity of the Soluble Specific Substances.

The unique relationships of the two species of bacteria afforded an opportunity of analysing the nature of complex antigens in terms of the type specific carbohydrate - or Soluble Specific Substance - and the species specific protein. In this way, light might be thrown upon the relative importance of these two cell constituents in the determination of type specificity. Accordingly observations were made on the serological relationships existing between the polysaccharide and protein, and between the encapsulated "S" cells and capsule-free "R" cells of Pneumococcus Type II and Friedlander's bacillus, Type B.

The results of this study show that the encapsulated (S) cells of both species are agglutinated in anti-S sera of either organism. The capsule-free cells (R) on the contrary are different for each species and react only with immune serum produced by the injection of pneumococci or Friedlander bacillus, respectively. It is significant in this connection that S cells differ from the corresponding R cells only in the possession of capsules and the characters which accompany capsules, such as virulence, type specificity and elaboration of Soluble Specific Substance. In other words, these two organisms of bilogically remote species, when encapsulated are endowed with similar immunological characters; but when they are devoid of capsules, they act as different and unrelated antigens. Since, as has been shown previously, the type specific carbobydrates are chemically and serologically similar, this fact constitutes direct evidence, that the immunological identity of encapsulated bacteria depends upon the chemical nature of this capsular substance.

It was not possible to show any serological relationship between the proteins of the two species by cross precipitation reactions. This fact, together with the lack of reciprocal relations between the degraded R strains derived from both species (Table I) throws the burden of type specificity of complex antigens on the Soluble Specific Substance. In other words, it appears that the proteins of encapsulated bacteria play little or no part in type specificity and that this property resides in the capsular or carbohydrate constituent of the cell. It further appears probable that when the analogous specific polysaccharides of otherwise totally unrelated microorganisms correspond sufficiently in chemical constitution an immunological correspondence also results. Moreover, the present study lends supporting evidence to the opinion

Cross agglutination of "S" and "R" strains of Pneumococcus Type II and Friedlander's bacillus Type B in anti-S and anti-R Sera.

	Strains	Antipneumococcus serum - Type II								Anti-Friedlander serum - Type B							
		Anti-S serum				Anti-R serum				Anti-S serum				Anti-R serum			
		1:5	1:10	1:20	1:40	1:5	1:10	1:20	1:40	1:5	1:10	1:20	1:40	1:5	1:10	1:20	1:40
S	Pneumococcus Type II	++++	++++	++++	++++	-	-	-	-	++++	+++	++	+	-	-	-	-
S	B.Friedlander Type B	++++	++++	+++	+	-	-	-	-	++++	++++	++++	+++	-	-	-	-
R	Pneumococcus Type II	±	-	-	-	++++	++++	++++	+++	-	-	-	-	-	-	-	-
R	B. Friedlander Type B	-	-	-	-	-	-	-	-	-	-	-	-	++++	++++	++++	++++

previously expressed that the type-specific character of the antigenic response is dependent almost entirely on the nature of the polysaccharide and not upon the substance to which it is attached. Consequently since the specific carbohydrate substance of the Friedlander Type B, and Pneumococcus Type II possess in common similar chemical properties the type specificity and antigenicity of each is similar even though, as the present work shows, the proteins in each instance are quite dissimilar.

Studies on Immunity to Pneumococcus Mucosus (Type III). (Dr. Tillett). In two publications now in press, certain phenomena with regard to the antigenicity and infectivity of Type III pneumococci for rabbits have been described. By way of recapitulation, the results of those experiments will be briefly summarized;- It was found that the immunization of rabbits with Type III pneumococci failed in a great majority of instances to stimulate the production of type specific antibodies, but was effective in producing antibodies, reactive with nucleo-protein common to all pneumococci, and capable of agglutinating any R strain of pneumococcus. These results are interpreted as meaning that rabbits possess some mechanism which is capable of altering the antigenic complex of the Type III pneumococcus so that the type specific component is made ineffective. Since type specificity resides in the soluble specific substance of the capsule, it seems evident that the injury inflicted on the organisms involves the capsule. Further evidence in support of this interpretation of the immunological response of rabbits is brought out when living Type III pneumococci are injected. It was found that these organisms, although highly pathogenic for mice and possessed of large mucoid capsules, are

avirulent for rabbits in doses of 2 cc. to 5 cc. and some times 10 cc. Since encapsulation and virulence are known to be intimately associated, it seemed possible that the method whereby rabbits rendered the Type III avirulent rested on the same mechanism which is responsible for the destruction of the type specific antigenicity of the cell, and that as a result of the injury inflicted on the capsule of the living organism, virulence is lost.

An attempt to understand the method whereby rabbits dispose of encapsulated Type III pneumococci intravenously injected led to a study of the bacteremia produced; and for purposes of comparison non-encapsulated "R" forms of pneumococci were also employed. Neither of these organisms produce fatal infection in rabbits. The promptness with which R forms are phagocyted following introduction into the circulation affords adequate explanation for the rapid disappearance of these organisms. However, the encapsulated "S" forms are not phagocyted and the bacteremia following injection of organisms of this character runs an uneven course which persists for several days before complete disappearance. A comparison, then, of events following injection of "R" and "S" forms of Type III pneumococci show striking differences. The "R" forms are readily phagocyted and disappear in a few hours. The "S" forms are not phagocyted in their encapsulated state and they persist in the blood stream for several days before final recovery of the animal. It is necessary, therefore, to ascribe the natural resistance of rabbits to Type III pneumococci to some, as yet, undetermined factor.

Of the strains of Type III used in these experiments one was made highly virulent for rabbits by means of rapid animal passage.

The bacteremia produced by this strain was characteristic of the septicemic curve, previously described by others, which consists in a sharp decrease in the number of circulating organisms followed by a steady increase until the death of the animal. This strain is identical in every other respect with the rabbit-avirulent strains of Type III. Therefore, avirulence must rest upon some acquired property by means of which the bacteria protect themselves against the resistance factors of the host. Further evidence in support of this is brought out by the fact that immunization of rabbits with the virulent strain results in the production of type specific antibodies.

Active Immunity against Infection with Rabbit-Virulent Strain of Type III Pneumococcus. Rabbits immunized with type specific pneumococci (Types I, II, and III) and "R" strains derived from the three fixed types were tested for active immunity against infection with the rabbit virulent strain of Type III. The immunized animals used in the experiments may be divided into 4 groups according to the organisms used for antigenic and the resulting antibody response.

Group I. Immunized with Type III pneumococci (Rabbit Avirulent Strains). Sera of only 3 out of 24 of the animals acquired both type specific antibodies and species specific antibodies - usually designated as anti-P - which are reactive with the common pneumococcus nucleo-protein and with any R form of pneumococcus. The sera of the remaining animals of this group acquired only anti-P antibodies.

Group II. Immunized with Type I or Type II pneumococci. Sera possessed homologous type specific antibodies and also anti-P antibodies.

Group III. Immunized with "R" forms of pneumococci. Sera possessed only anti-P antibodies.

All of these immunized rabbits were infected intravenously with 1 cc. of the rabbit virulent strain of Type III. Appropriate controls were used in each test. Usually .0001 cc. or.001 cc. of the virulent culture was fatal for normal rabbits and .01 cc. was always fatal. Normal rabbits receiving 1 cc. of culture succumbed in 16 to 36 hours. In many instances the bacteremia was studied by means of blood cultures taken at frequent intervals in order to determine the duration and intensity of the blood infection. The striking fact observed in these experiments is that in rabbits active immunity against Type III pneumococci may be induced by immunization with pneumococci of a heterologous type or with "R" strains. Of 26 rabbits so immunized, 18 (69 per cent) survived; 6 other animals lived 6 to 12 days after the controls had died. Moreover, in each instance the bacteremia was much less severe, and at autopsy they all showed a fibrino-purulent pericarditis and pleurisy. These animals were considered as showing definite evidence of increased resistance. If they are included with the rabbits which survived, 24 or 92 per cent of this series showed evidence of active immunity against infection with Type III organisms. Of the 12 rabbits immunized with Type III, and only 3 of which possessed demonstrable type specific agglutinins, 6 (50 per cent) survived and 4 others showed evidence of increased resistance, although ultimately succumbing to a localized pericarditis and pleurisy. In this series 10 or 91 per cent showed evidence of active immunity.

The results of these experiments show definitely that rabbits may be actively immunized against infection by avirulent Type III pneumococcus although type specific antibodies are not present in the circulating blood. Indeed, the animals possessing type specific agglutin-

ins showed no higher degree of immunity than those without them. An explanation of the immunity of those rabbits immunized with Type III but possessing no demonstrable type specific agglutinins in their serum might possibly rest upon the fact that the specific antibodies were sessile. However, all possibility of a type specific reaction is excluded in the animals immunized with Type I and II and R forms and subsequently infected with a virulent strain of Type III. In these animals the active immunityis as effective as in those immunized with Type III. It may be noted that all the rabbits possessed anti-protein antibodies. All previous work has shown that this type of antibody confers no passive protection. None of the sera of the immunized rabbits used in these experiments conferred passive protection on mice except those possessing type specific antibodies. Further evidence of the ineffectiveness of anti-P anti-bodies in active immunity is brought out by immunization with solutions of pneumococci. This process results in the production of anti-P antibodies but not in active immunity.

From the experiments described it seems highly improbable that demonstrable antibodies are of significance in the active immunity of rabbits agains infection with Type III pneumococci. It is possible, however, that an adequate explanation may be found in the natural resistance of rabbits to Type III pneumococci. The experiments summarized in the first part of the report on immunization and infectivity show that rabbits possess the ability to destroy encapsulated Type III pneumococci. Since rabbit sera contain no substances capable of damaging these organisms in vitro and since rabbits' leucocytes are incapable of phagocyting encapsulated pneumococci, the natural resistance must reside in some factor whose nature is, as yet, undetermined but whose

presence must be admitted. It is not improbable that this factor (natural resistance) is amenable to stimulation and that when it is exalted there results increased natural resistance and that on this reaction active immunity depends. The conception is, therefore, advanced that the increased resistance to infection with Type III pneumococcus which may be induced in rabbits by non specific means rests not on the production of some new factor - as antibodies - but upon the heightened activity of those factors already present in the animal body which endow the rabbit with natural resistance to Type III pneumococci.

Chemistry of Soluble Specific Substance. (Dr. Heidelberger). (In the autumn Dr. Heidelberger was asked to take charge of the Chemical Laboratory at the Mount Sinai Hospital which invitation he accpted and on February first he left us to take up his new duties. Up to the time of his departure he was busily engaged in completing work on the problems concerning the Soluble Specific Substance which were already under way and the nature of which has been discussed in previous reports. The following notes by Dr. Heidelberger indicate the new observations which were made before his departure.)

In the case of the soluble specific substance of Type I pneumococcus it was found that on energetic oxidation as much as one-third of the substance could be recovered as mucic acid, indicating that galactose or galacturonic acid units make up possibly as high as 50 per cent of the specific substance. A clue to the remaining portion is furnished by the presence also of an optically active acid oxidation product which has not yet been studied in detail. It is hoped to continue in the Mount Sinai chemical laboratory studies on the Type I ma-

terial already on hand and publish any results that may be obtained as a joint communication from the two hospitals.

The difficulty of obtaining pneumococcus material led to a further study of the soluble specific substance derived from gum arabic, in which the results given in a previous report were confirmed and sugar acids of a type found in the Type III pneumococcus substance again isolated.

From a sample of lemon pectin in the laboratory a fraction was also isolated which reacted with Type II and Type III anti-pneumococcus sera, but as the amount recovered was very small, and pectin is made from lemons contaminated with mould, the origin of the specifically reacting carbohydrate remains in doubt.

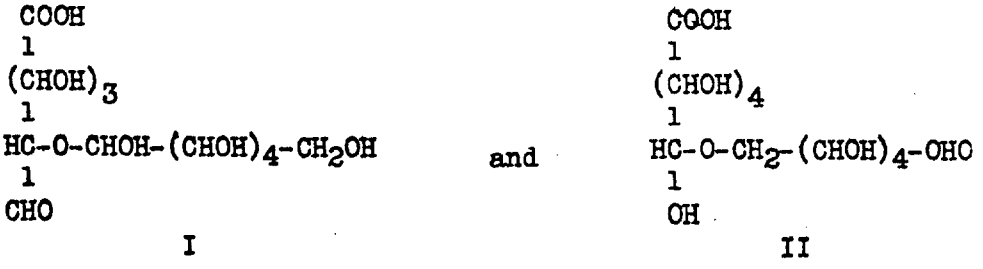
Chemistry of the Soluble Specific Substance of Pneumococcus and Friedlander Bacillus. (Dr. Goebel). A. Methods. 1. During the course of an investigation on the chemical nature of an aldobionic acid isolated as a hydrolytic product from the specific polysaccharide produced by pneumococcus Type III during growth, it became necessary to oxidize this aldehydic sugar as a step in the elucidation of its structure. For purposes of oxidation the method devised by Emil Fischer, and which has been generally used, failed. A new method for converting any aldose unfailingly into the corresponding sugar acid has been devised. The method embodies the principle expressed in the equation : $2RCHO+3Ba(OH)_2+2I_2=(RCOO)_2Ba+2BaI_2+4H_2O$, and possesses the advantages of speed, of freedom from technical difficulties, and finally of bringing about the conversion aldose ---+ sugar acid practically quantitatively. By this method gluconic, maltobionic, lactobionic and "saccharobionic" acids have been prepared. During the course

of this research it was observed that aldoses are not quantitatively oxidized to the corresponding sugar acid if the initial concentration of hydroxyl ions is too great. A theorectical discussion which attempts to explain this phenomenum has been submitted for publication.

2. The general method hitherto of separating sugar acids from true sugars in the hydrolytic products of sugar acid-containing polysaccharides makes use of the fact that sugar acids form an insoluble lead salt. Thus, when one treats a mixture of sugar acids and sugars with basic lead acetate, a separation may be obtained, for the sugar acid lead salt is precipitated and the sugars remain in solution. This separation is by no means quantitative for a relatively large part of the sugar acid lead salt remains in solution with the sugars. It is therefore difficult to investigate the nature of the sugar fraction since it is contaminated with sugar acid. It is furthermore extremely difficult to obtain any precise knowledge of the ratio of sugar acid to sugar since a quantitative separation cannot be effected. A new technique has therefore been devised which consists in boiling the hydrolytic product of acidic polysaccharides with calcium carbonate. All of the sugar acids are thus converted into the calcium salts and the non-acidic true sugars remain unaltered. On evaporating the mixture and on the subsequent addition of methyl alcohol (in which the calcium salts of sugars are insoluble) the sugars go into solution and may be quantitatively separated from the sugar acid calcium compound. This method of separation is of importance for it permits the isolation of small quantities of sugar acids which would otherwise be lost and because it permits a quantitative analysis of the ratio of sugar to sugar acid - an important point in the elucidation of the structure of

the acidic polysaccharide molecule.

B. Type III Pneumococcus. It has formerly been reported that the aldobionic acid $C_{11}H_{19}O_{10}$ COOH forms by far the major portion of the hydrolytic products of the pneumococcus Type III specific polysaccharide and evidence has been presented for the belief that this specific substance may be grossly represented by the expression (aldobionic acid)$_x$. Since this compound is apparently the fundamental building stone of this carbohydrate, a detailed study of the aldobionic acid has been made. By means of the new technic described above it has been possible to secure this material from the hydrolytic products of the polysaccharide in a high state of purity and in greater yields than hitherto. The sugar is composed of one molecule of glucose combined through glucosidic linkage to one molecule of sugar acid of the glucuronic type. Of the numerous possible structures of this compound the two most logical may be represented by the formulae



I II

By means of barium hypoiodite the aldobionic acid has been oxidized to the corresponding "saccharobionic" acid and a calcium salt, having the proper analytical constants, has been isolated. This calcium salt gives a strong naphtho resorcinol test and yields 17% of furfural on distillation with 12% HCL. If formula I were the correct formula for the original aldobionic acid, the oxidation product, saccharobionic acid, would obviously contain one molecule of glucose and one molecule of saccharic acid in glucosidic linkage, and

there would be no substance capable of yielding furfural on distillation with hydrochloric acid, within the molecule. On the other hand, in formula II, the uronic acid would remain unimpaired on oxidation to the saccharobionic acid since the glucosidic linkage is through the aldehyde group of the uronic acid and the resultant saccharobionic acid would be a compound of gluconic acid and a uronic acid. This compound would, of course, yield furfural. In view of the experimental observations formula II must be assigned to the aldobionic acid. This compound is unique in the field of sugar chemistry.

Although a great deal of indirect evidence has been gathered which points to the assumption that the sugar acid half of the aldobionic acid molecule is glucuronic acid, no direct evidence (i.e. no derivative of glucuronic acid itself) has ever been obtained. This is due to the fact that the aldobionic acid is an extremely stable compound and only very prolonged boiling with a mineral acid will effect hydrolysis. Under such conditions acids of the glucuronic type are decomposed, and it is impossible to prepare any derivative.

It was, however, thought possible to hydrolyze this extremely stable compound by means of dry methyl alcoholic hydrochloric acid. If this treatment is effective one should obtain as products of hydrolysis the methyl glucoside of glucose and the methyl glucoside of glucuronic methyl ether. The two compounds could be readily and quantitatively separated by warming with barium hydroxide and pouring the mixture into alcohol, and the end products could be readily identified as their osazones after hydrolysis of the glucosides. This investigation is at present under way, and promises to give definite direct evidence as to the exact nature of the uronic acid fraction of the aldo-

bionic acid molecule.

C. Type II Pneumococcus. It has been found that the Soluble Specific Substance of Type II pneumococcus contains a sugar acid of the glucuronic type and that this specific soluble substance bears a chemical analogy to that of Type III Pneumococcus.

D. Type A Friedlander bacillus. The soluble specific substance from Type A Freidlander bacillus is apparently constituted from three distinct sugars. The first is a sugar acid either identical or isomeric with that obtained from Pneumococcus Type III soluble substance, the second is glucose which has been identified by oxidation to saccharic acid and as its osozone. The third sugar is one which appears together with glucose in the true sugar fraction of the polysaccharide's hydrolytic products. This sugar may be separated from glucose by fermenting away the latter with yeast. It contains no glucuronic acid derivative, nor is it a pentose or pentose derivative. It yields a precipitate on boiling with barium hydroxide, and it forms no typical osazone. This sugar has not as yet been identified. The Friedlander Type A soluble specific substance itself is a white amorphous powder, soluble in water. It has an acid equivalent of 460, it rotates the plane of prolonged light 101° to the left, it gives 60% reducing sugar on hydrolysis (calculated as glucose). It reacts with its homalogous anti-serum in dilution of 1:2,000,000.

E. Type B. Friedlander bacillus. By means of the technique described under A2 the calcium salt of a sugar acid either identical or isomeric with that obtained from the Type III soluble specific substance has been isolated from the hydrolytic products of the specific polysaccharide of Type B Friedlander bacillus. This sugar was not

found in the previous studies on this polysaccharide. A quantitative study of the hydrolytic products reveals the fact that the polysaccharide is built up from glucose and a sugar acid of the glucuronic acid type in a ratio of 3 glucose molecules to 1 uronic acid molecule. The Pneumococcus Type III soluble specific substance has been shown to be constituted from glucose and a uronic acid molecule in a ratio of 1:1. This specific polysaccharide may roughly be represented by the formula $(\text{glucose-uronic acid})_x$; similarly the Type B Friedlander specific polysaccharide may be represented by the formula $\left[\begin{matrix}\text{glucose}\\ \text{glucose}\end{matrix}\right>\text{glucose} - \text{uronic acid}\Big]_x$. Such a compound should theoretically have an acid equivalent of 682 and a carbon and hydrogen content of 42.2 and 6.2 per cent, respectively. These figures are in close analogy with those found experimentally. Thus a close analogy between the polysaccharides of these widely different organisms is to be seen. It is hoped that this analogy may, in a similar manner, be extended to the polysaccharides of the other organisms under study.

F. Type C.Friedlander bacillus. By methods essentially the same as those used in the case of the soluble specific substance of Pneumococcus and Type A and B Friedlander bacillus a nitrogen free polysaccharide with specific properties was isolated from the Type C Friedlander bacillus. It is a white amorphous powder having an acid equivalent of 480 and is soluble in water and alkali. It reacts with its homologous antiserum in dilutions of 1:2,000,000. It rotates the plane of polarized light 100° to the right. It yields glucose and a sugar acid on hydrolysis. The isolation of this substance concludes a systematic investigation of the specific polysaccharides of the three fixed types of Friedlander's bacillus.

It is hoped ultimately to show that the specific polysaccharides both of the fixed types of Pneumococcus and of Freidlander bacillus bear a distinct and extremely close chemical analogy to one another in regard to the building stones, the sugars and sugar acids, which enter into the network of the complex polysaccharide molecule, but that the definite chemical and immunological differences displayed by the various polysaccharides depends first on the ratio of sugar to sugar acid which go to make up the complex molecule and second on the position of chemical linkage of sugar to sugar within the polysaccharide molecule itself.

Observations concerning the Reversion of "R" Cultures of Pneumococcus to the "S" Type. (Drs. Dawson and Avery). The question of the reversion of "R" cultures of Pneumococci - avirulent, non-type specific, having no demonstrable capsule and elaborating no specific soluble substance, - to the "S" type, - virulent, type-specific, capsulated, and producing the specific soluble derivative, - has been the subject of further investigation.

Animal Passage and Cultural Methods have been employed and the following results obtained: -

A. Animal Passage. By repeated rapid passage in mice "R" cultures of Type II and Type III (Laboratory strains D/39/R and M/3/R have been caused to revert to the "S" type, having all the properties characteristic of the homologous type-virulent, type specific, capsulated and "S" producing.

As yet it has been found impossible to revert the Type I R strain which has been employed (Laboratory strain 1/192/R- Neufeld). Efforts are being made to revert other Type 1 R strains by this method.

B. Cultural Methods. Inasmuch as it is well known that one of the best methods to effect the transformation of "S" cultures to the "R" type is to grow the "S" organisms in media containing Anti-S antibodies, (i.e. homologous immune serum), it was thought possible that the reverse process might be initiated by growing "R" organisms in media containing anti-pneumococcus-protein (anti R) antibodies. Accordingly Types I R, II R, and III R, cultures (I/192/R, D/39/R, M/3/R) were grown in 10% anti-protein rabbit serum. After six to eight transfers the change was effected with Types II R and III R but so far attempts to revert the I R culture have failed. Other I R strains are now being used.

At this time it was shown in connection with other work that normal human sera contained anti-antibodies. It was therefore suggested that "R" cultures might revert to "S" cultures if human sera were employed. Similar results were obtained. In like manner it was also found possible to effect the change by using immune horse-sera of heterologous types, which contain in common the pneumococcus-protein antibodies. Two questions then arose: -

(1) Do all the cells of a given R strain possess the ability to revert in the cultures employed or only certain ones?

(2) Is reversion due to the presence of the specific anti-protein antibody stimulus or is it simply dependent upon nutritional factors supplied by the use of serum-broth?

The first question has been answered by employing pure line strains derived from single-cell cultures. Two single-cell cultures of II R (D/39/R) and four of III R (M/3/R) were used and reversion effected in each instance. Reversion by cultural methods is always ac-

companied by the acquisition of virulence so that 0.00001 cc. of the reverted culture kills white mice in 24 hours, whereas the original "R" strain fails to kill in doese of 1 cc.

The second question is now being investigated by employing serum-broth free of anti-protein antibodies. At present the work is incomplete but it is possible that the change may also occur, but less readily, in the absence of anti-protein antibodies.

Throughout the work the quality of the media has been found to be of paramount importance. Media rich in growth promoting factors has given results where routine media has failed.

From the foregoing it would appear that the great majority, if not all, of avirulent "R" cells have the ability, under proper environmental conditions, to revert to virulent, type-specific capsulated organisms.

Antigen - Antibody Balance in Pneumonia. (Dr. Dawson). The object of the work undertaken was, generally stated, to follow the antigen - antibody balance in patients suffering from lobar pneumonia. More especially the problem related to the demonstration of the anti-protein antibody response during the course of the disease, inasmuch as the antigenic properties of the intact cell and the antibody response of the human body to the intact cell and its soluble derivative had already been well established.

Early during the work it became apparent that the anti-protein antibody response did not show a wide range of variation. Preliminary work on immune animal sera indicated that the Thread Reaction would be the most suitable method for its detection.

Serial bleedings were taken on a series of patients with lo-

bar pneumonia and the sera were tested by the thread reaction. Observations made on a series of twelve cases suggest that little, if any, change occurs in the content of anti-protein antibody. Those cases in which the sera were tested by agglutination with heat-killed "R" cultures also failed to show an increase in this anti-body. It is now proposed to confirm this finding by precipitin tests using autolyzed "R" cultures and nucleo-protein as precipitinogen.

The elaboration of the soluble derivative of the pneumococcus was followed in these cases and the appearance of an antibody for this substance at or about the time of crisis demonstrated.

The sera of a series of normal adult individuals were also examined for the presence of the anti-protein antibody. With the methods employed it was indicated that this antibody was present in every instance.

While this problem was being investigated some work was also done on the reversion of the "R" type of pneumococcus to the "S" type. Following Dr. Avery's observation that "R" cultures, Types II and III, would revert to "S" cultures, under certain circumstances, when grown in "anti-R" sera, it was found that the same transformation would occur when type-specific horse serum of heterologous type was employed. The possibility of the phenomenon occurring in normal human sera was suggested by the demonstration of the anti-protein antibody in these sera. Experimental attempts to effect the change in this way accordingly were made and have, in certain instances, also met with success. It is now proposed to demonstrate whether the transformation is simply the result of nutritional factors or whether it is due to a specific antibody stimulus.

Culture Media - Miss Holt (Report by Dr. Avery). During the past twelve months the culture medium supplied to the Bacteriological Laboratories has been very unsatisfactory. Casual attempts to improve the media by changing the quality of the meat and the other ingredients, such as peptone, salts, etc. have been wholly without success. Suggestions from the members of the Staff and the cooperation of the Media Department have likewise failed to improve the situation. It seemed desirable, therefore, to make a thorough study of the factors which determine the growth promoting qualities of culture media, since obviously principles more subtle than mere content of nitrogenous material, the proper salt balance, and the optional hydrogen ion concentration are involved. To this end Miss Holt, working in the bacteriological laboratories of the Hospital, is engaged at present in an attempt to analyze those factors which are requisite for growth, and which satisfy the nutritional needs of the bacteria. The evidence available at present indicates that the factors requisite for the initiation and maintenance of growth are of the nature of substance which meet certain physiological needs of cell and which are not necessarily furnished by the food stuffs utilized in the processes of cell metabolism. These factors, although present to a greater or less extent in the original materials, are lost in the process of filtration, or are actually destroyed by boiling and by final sterilization of the medium at an alkaline reaction. These factors may be represented by the expression $C+[V+X]$, in which "C" represents an essential though slight concentration of a readily utilizable carbohydrate, which furnishes a ready source of energy for the initiation of growth; and $[V+X]$ represents a physiologically active system which has previously been shown to be

essential for the growth of organisms of the so-called hemophilic group.

In meat, the C factor is present as native muscle and blood sugar, but naturally varies in concentration depending upon the nutritional state of the animal before slaughter, and upon the amount of glycolysis and oxidation which takes place during subsequent handling of the meat. Consequently the "C" factor is a variable which can be determined for any given lot of medium by fermentation tests and chemical methods. This variation can be overcome by the addition of very small amounts of dextrose (0.02 - 0.05%) - a concentration found to be optional for initiation of growth but insufficient to produce the detrimental acidity which always accompanies the metabolism of larger amounts of sugar.

The "V" factor" is a term used to designate these substances which are vitaminelike in nature and which correspond to "bios". This factor, always present in fresh plant and animal tissues, functions in a remarkable way in stimulating bacterial growth. The present studies indicate, however, that this growth-accessory substance is completely destroyed when heated at the pH and for the period which corresponds in alkalinity and time to the process to which media are subjected by present methods of preparation. By reason of the known role of "V" factor in the growth of B. influenzae this organism serves as a delicate biological indicator of the presence or absence of this factor in any given lot of media. By methods previously worked out extracts of plant cells (tomatoes and yeast) which are rich in V content, are now available and utilizable in this experimental work. Used as enrichment fluid these extracts have an extraordinary growth-promoting in-

fluence when added to an otherwise unfavorable medium.

The "X" factor, which acts as a bio-catalyst, is of importance in the oxidation-reduction processes of the cell. It has the functions of a peroxidase, and to it has been attributed a role in oxygen transport. As is well known, its presence is essential in the cultivation of hemophilic bacteria, and, while not requisite for growth of pneumococcus, it apparently exerts a definite action in conserving the viability of cell. It is known to prevent the accumulation of peroxide which always occurs in culture fluid when pneumococci are grown in the presence of oxygen. This substance, related to the active iron salts present in plant and animal tissue, although heat stabile, is lost by filtration, being carried out of solution by adsorption to the heat and coagulable proteins during the preparation of media.

Attempts are now being made to conserve, as far as possible, these factors as they occur naturally in meat, and to make up their deficiency by the addition of suitable substitutes to the final media. Methods are also being tested which will permit of the application of these principles to the mass production of media.